Scanning Microscopy

Volume 5 | Number 1 Article 18

12-23-1990

Cytology of Pollutant Metals in Marine Invertebrates: A Review of Microanalytical Applications

J. A. Nott
Plymouth Marine Laboratory

Scanning Microscopy, Vol. 5, No. 1, 1991 (Pages 191-205)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/91$3.00+.00

CYTOLOGY OF POLLUTANT METALS IN MARINE INVERTEBRATES: A REVIEW OF MICROANALYTICAL APPLICATIONS

J.A. Nott

Plymouth Marine Laboratory, Citadel Hill, Plymouth,
Devon PL1 2PB, U.K. Tel: 0752 222772

(Received for publication May 6, 1990, and in revised form December 23, 1990)

Abstract

X-ray microanalysis (XRMA) is customized for investigations of the metabolic and detoxification strategies of heavy metals taken by marine organisms from polluted environments. Sites of uptake, intracellular accumulation, transport and excretion are visualized, analysed and quantified. Cryopreparation techniques are required to prevent the translocation or loss from specimens of soluble metal species.

In marine invertebrates, metals are detoxified by systems of chemical binding and intracellular compartmentalization. XRMA investigations have concentrated on marine molluscs and crustaceans and even within these restricted groups there are marked inter-species differences in the biochemical and cytological processes which reduce metal bioavailability. Some detoxification systems also protect the carnivores which ingest the metal-laden tissues of the prey. This results in the bioreduction of metals along a food chain. These processes are investigated by XRMA which can be tuned to observe the complex interactions which operate at all levels within and between the biota and polluted environments.

KEY WORDS: X-ray microanalysis, cryofixation, marine invertebrates, marine pollution, metals, metal bioavailability, metal detoxification (detoxication), phosphate granules, sulphur ligands

Introduction

Before the development of X-ray microanalysis (XRMA) the metabolism of metals was investigated by histochemistry, toxicity testing and quantitative analyses of tissues and whole animals by atomic absorption spectrophotometry. It was established that metals were accumulated within organisms, sometimes bound to metalloproteins and that there were varying degrees of tolerance. Limited progress was made towards understanding the cytology and the inorganic biochemical mechanisms involved. The application of XRMA produced spectacular advances because it revealed the intracellular location of different metals and the binding ligands. The technique is customized for this area of research and various applications have been cited in earlier reviews (Table 1-4).

In this review, XRMA means the use of a solid state detector in conjunction with the scanning electron microscope (SEM), scanning transmission electron microscope (STEM) and transmission electron microscope (TEM). These systems became available in the early 1970's and applications to the cytology of metals in marine invertebrates were soon described (Nott and Parkes, 1975; Walker et al. 1975a & b).

Metals bind with a restricted range of ligands and generate a cascade of reactions that can affect biochemical processes, structure and function of cells, tissues and whole organisms and individual behaviour as well as population dynamics. The reactions are chemically simple but biologically complex. In terms of physical chemistry, metals can be grouped into the hard acid type, Na, Mg, K and Ca which bind electrostatically with a ligand preference in the series O N S and the soft acid type Cu, Cd, Hg and Ag which bind covalently with a preference for S N O. Other metals including Mn, Fe, Co, Ni and Zn show intermediate properties. Most metals, including both those essential and non-essential for normal metabolism, are toxic when encountered in excess quantities.

Instrumentation

During the period 1970-1990, XRMA equipment became rapidly more sophisticated and techniques of specimen preparation were developed which reduced the loss and redistribution of elements within tissues. The first solid state detectors were attached to SEMs and transmission detectors

for use in SEMs (STEM) had to be fabricated in-house for viewing and analysing. Later, detectors were attached to TEMs with STEM attachments; these systems had collimated beams and small spot sizes and produced high resolution images and low background X-ray spectra. Modern microscopes have features which are dedicated to XRMA and these range from the design of the specimen chamber to enable efficient X-ray detection to integration of the control system of the electron microscope and analyser.

Specimen preparation

XRMA of metals in tissues requires special preparation techniques. Early work used standard EM chemical fixation and dehydration but avoided osmium post-fixation and copper grids to reduce unwanted X-ray peaks. These techniques removed the labile physiological elements, Na, K, Cl and Mg, and the non-essential pollutant element, cadmium. Only the most insoluble, intracellular metal species were retained. Some specialized fixatives were developed which contained pyroantimonate, H_2S and other reagents for the precipitation of metals within cells. These methods were unsatisfactory because they were non-specific, there was translocation of the elements and additional, major X-ray peaks derived from fixatives obliterated some of the important peaks from metals and ligands.

At the present time, chemical techniques have been replaced by cryo-methods; fresh tissue is quenched rapidly in liquid ethane or other cryogen cooled by liquid nitrogen (Ryan and Purse, 1984, Ryan et al. 1987, 1990) and analysed in the hydrated or dehydrated state. Specimens are dehydrated by freeze-drying or freeze-substitution. Hydrated specimens present the ultimate preparation but requirements for cryo-ultramicrotomy and EM cold stages tend to reduce the output of analytical data. It can be argued that drying techniques induce some translocation of elements but an acceptable compromise to achieve rapid output of data and meaningful results can be achieved by freeze-drying for analysis and freeze substitution for structure (Nott and Nicolaidou, 1989b). The distribution of metals in sections can be demonstrated spodographically by micro-incineration which removes all organic material and retains a cytological image formed from inorganic ash (Mason and Nott, 1980; Al-Mohanna and Nott, 1986a). This technique also increases the mass fraction of metals in sections and has enabled labile cytosolic zinc to be detected in the nephrocytes of the winkle (Mason and Nott, 1980).

Intracellular pollutant metals occur in the cytosol, membrane-bound vesicles and mineralized concretions. These compartments are structured and, therefore, do not have a 'matrix' with a meaningful mean atomic number to provide the basis of metal concentrations in fully quantitative analyses. Accurate analyses of solubilized tissue can be determined from micro-droplets (Nott and Mavin, 1986) but this does not provide information on intracellular distribution. However, there are numerous examples in the literature where semi-quantitative analyses have determined the ratios of metals to phosphorus and sulphur which are the two intracellular XRMA markers for phosphate and SH-metal ligands (see Tables 1-4 for references).

Metal detoxification in tissues

XRMA has shown that metals in marine invertebrates are generally localized in membrane-bound granular accumulations which are confined to particular cell types. There are cytosolic metallothioneins which bind metals like cadmium, zinc and copper but these proteins are probably degraded in the lysosomes where the metals remain in association with sulphur (see Table 4 for references). Various combinations of intracellular chemical binding and compartmentalization serve as detoxification systems which protect normal biochemical processes from disruption by reactive metals. Furthermore, detoxification systems tend to confine the metals to particular tissues.

The marine invertebrates which have received the most XRMA investigation are gastropods, including winkles and whelks, bivalves, including clams, oysters and mussels and crustaceans, including lobsters, crabs and shrimps. A summary of sites of uptake, translocation, storage and excretion of metals by these animals is given in Figs 1-3 and references to the respective XRMA investigations are given in Tables 1-3. Reports which include XRMA results on other marine animals are listed in Table 5.

The diagrams show that metals are taken up by animals at several sites, particularly the gills and gut. They are transported by blood cells and accumulated in the gut of crustaceans and gastropod molluscs and in the kidney of bivalve molluscs. They are excreted via the gut, kidney and other tissues. The biology of metals is essentially dynamic and any assessment of the metal content of an animal represents the net result of continual uptake and loss.

XRMA has shown graphically the uptake of iron into membrane bound pinocytic vesicles in the gills and gut of mussels (George et al. 1976) and there are descriptions of amoebocytic blood cells transporting copper and zinc in membrane-bound granules in bivalves (see Table 2 - blood - for references).

The detoxification systems of binding ligands and compartmentalization operate on a considerable scale in digestive glands of marine gastropod molluscs and crustaceans and in kidneys of bivalve molluscs. It is in these tissues that the highest concentrations are accumulated and individual metals can exceed 1% of the dry mass. Moreover, during the course of cell turnover metal accumulations are lost along with other fragments of necrotic cells and this provides a system of excretion.

Direct XRMA observations using STEM and SEM (Nott and Nicolaidou, 1989a & b) have been made on three species of Mediterranean gastropod sampled offshore from a nickel smelting plant. The species are *Cerithium vulgatum* which feeds on sediments, *Monodonta* spp. which graze on algae and *Murex trunculus* which is carnivorous and includes *Cerithium* in its diet. The highest levels of metals are accumulated in the digestive gland of *Cerithium* where they occur in spherical mineralized granules in association with phosphorus Figs 4 & 5).

Toxicity reduction along a food chain

Cerithium granules contain metals which are not transferred to the carnivore *Murex*. This

Table 1. References to XRMA work on metals in marine crustaceans. (R = Review)

	Reference(s)	Metal(s)	Animal(s)	Tissue(s)
	Al-Mohanna & Nott, 1986a, b, 1987, 1989	Cu, Zn, Au, Th, Fe	Shrimp	Gut
	Chassard-Bouchaud, 1985	Cu, Zn, Mn, Fe, Fr, Cd, Ag, etc.	Shrimp	Gut
	Hopkin & Nott, 1979, 1980	Pb, Ca, P, Au, Th, S	Crab	Gut
R	Icely & Nott, 1980, 1985	Au, Fe, Th, Cu	Amphipod	Gut
	Koulish, 1976	Zn, P	Barnacle	Gut, body wall
	Nott & Mavin, 1986	Ca, Mg, P, S	Shrimp	Gut, Blood
	Nott et al., 1985	Pb (aryl sulphatase)	Copepod	Gut
R	Rainbow, 1987	Cu, Zn, Mn, Fe, etc.	Barnacle	Gut parenchyma
	Thomas & Ritz, 1986	Zn	Barnacle	Gut parenchyma
	Walker, 1977	Cu, Zn	Barnacle	Gut parenchyma
	Walker et al., 1975a, b	Zn, Mn, Fe, Z	Barnacle	Gut parenchyma

Table 2. References to XRMA work on metals in marine bivalves. (R = Review).

	Reference(s)	Metal(s)	Animal(s)	Tissue(s)
	Ballan-Dufrancais et al. 1982, 1985	Metals	Scallop	Gut
	Carmichael & Fowler, 1981	Cd	Scallop	Kidney
	Carmichael et al. 1979	Ca, Mg, P, Zn, Mn	Scallop	Kidney
	Chassard-Bouchaud, 1983	U, P	Mussel	Blood, kidney, gut
	Chassard-Bouchaud & Hallegot, 1984	La, P	Mussel	Gut macrophages, gill
	Chassard-Bouchaud et al. 1985, 1986, 1989	Ag, Pb, Cr, P, S	Mussel, oyster	Gut, gill, blood, muscle, kidney, byssus
R	Coombs & George, 1978	Fe, Pb, Cu, Zn, P, S	Mussel, oyster	Gill, amoebocytes, kidney
	Doyle et al. 1978	Ca, Mg, P, Mn, Fe, Cu, Zn	Clams	Kidney
	Fowler et al. 1975	Fe, Hg	Clam	Mantle
	George & Pirie, 1979, 1980	Mg, Ca, P, Zn, Fe, Cd	Mussel	Kidney, blood
	George et al. 1976, 1977, 1978, 1980	Fe, Zn, Mn, Ca, P, Cu, S	Mussel, scallop, oyster	Gill, blood, kidney, gut, amoebocytes
	Marshall & Talbot, 1979	Cd, Pb	Mussel	Gill
	Martoja & Martin, 1985	Cd, Zn, Cu	Oyster	Amoebocytes
	Martoja et al. 1985	Ag	Scallop	Gut
	Mauri & Orlando, 1982	Mn	Wedge shell	Kidney
	Pirie et al. 1984	Cu, Zn	Oyster	Blood
	Schulz-Baldes, 1977	Pb, P, S	Mussel	Blood, kidney
	Thomson et al. 1985	Cu, Zn, Fe	Oyster	Blood, kidney, mantle, gill

Table 3. References to XRMA work on metals in marine gastropods. (R = Review)

	Reference(s)	Metal(s)	Animal(s)	Tissue(s)
	Bouquegneau & Martoja, 1982	Cu, Cd, Zn	Gastropods	Gut
R	Bouquegneau et al. 1984	Metals	Molluscs	Gut
R	Greaves et al. 1984	Mn	Snail	Gut
	Martoja et al. 1980	Cu	Winkle	Kidney, gut
	Martoja et al. 1985	Cu, Ag	Whelk, winkle	Gut
	Mason & Nott, 1980	Zn, Mn, Fe	Winkle	Gut, kidney
R	Mason & Nott, 1981	Metals	Gastropods	Blood, gut, mantle, kidney, shell
	Mason & Simkiss, 1982	Mn	Snail	Gut
	Mason et al. 1984	Mn, Fe, Zn, Cu	Winkle	Kidney, gut, gill
	Nott & Langston, 1989	Cd, P, S	Winkle	Gut
	Nott and Nicolaidou, 1989a, b, 1990	Metals, Zn, Mn	Gastropods, whelks	Gut, faeces
R	Simkiss, 1979, 1984	Metals/granules, Mn	Molluscs/invertebrates	Gut
R	Simkiss & Mason, 1983, 1984	Metals, Cu, Fe, Mn, Zn	Molluscs, winkle	
	Taylor et al. 1988	Mn	Snail	

Figs 1-3. Schematic diagrams of marine organisms to show accumulations of metals in cells and the blood as detected by XRMA. Sources of the data in Figs 1-3 can be found by making cross references to the metals and tissues listed in Tables 1-3 respectively.

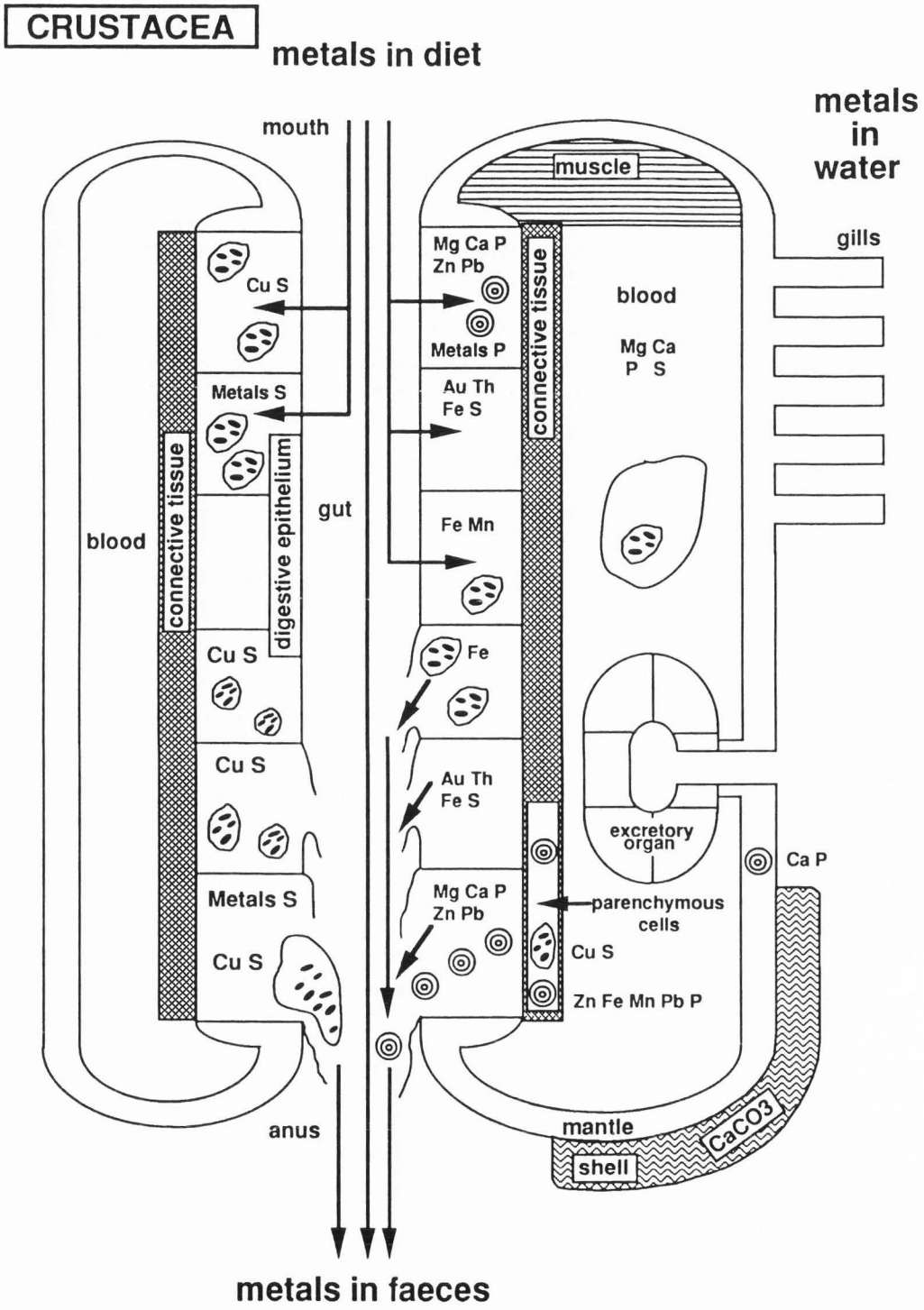

Fig. 1. Marine crustaceans. Sites of metal accumulation occur mainly in the epithelial and sub-epithelial cells of the gut. The gills, blood and excretory organs are active in the uptake, transport and excretion of metals (for example, Bryan et al., 1986) but no accumulations in these tissues have been detected by XRMA.

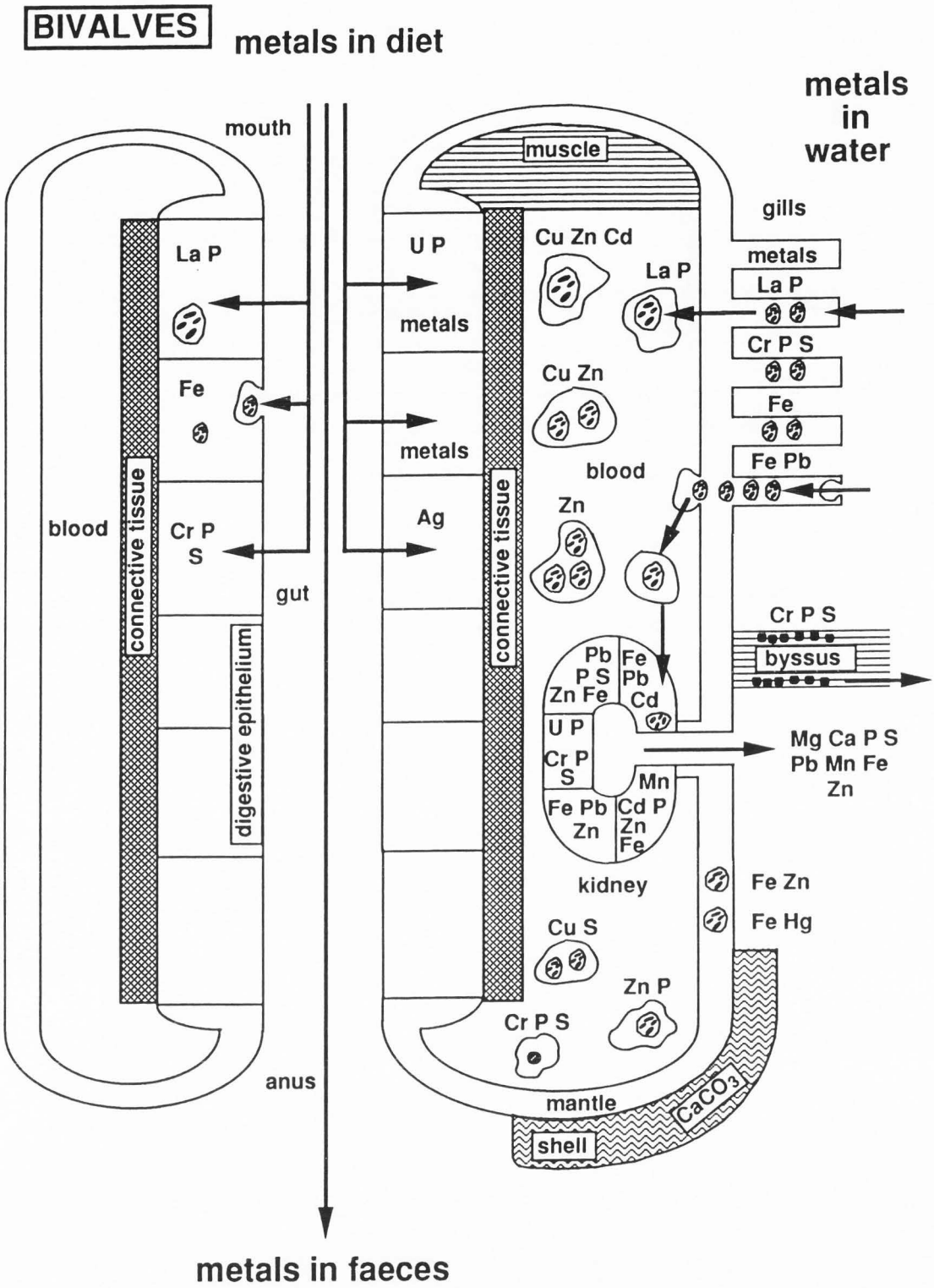

Fig. 2. Marine bivalves. Accumulations of metals take place at sites of uptake in the gut and gills. However, in these animals the kidney is the dominant tissue for accumulation and excretion of metals.

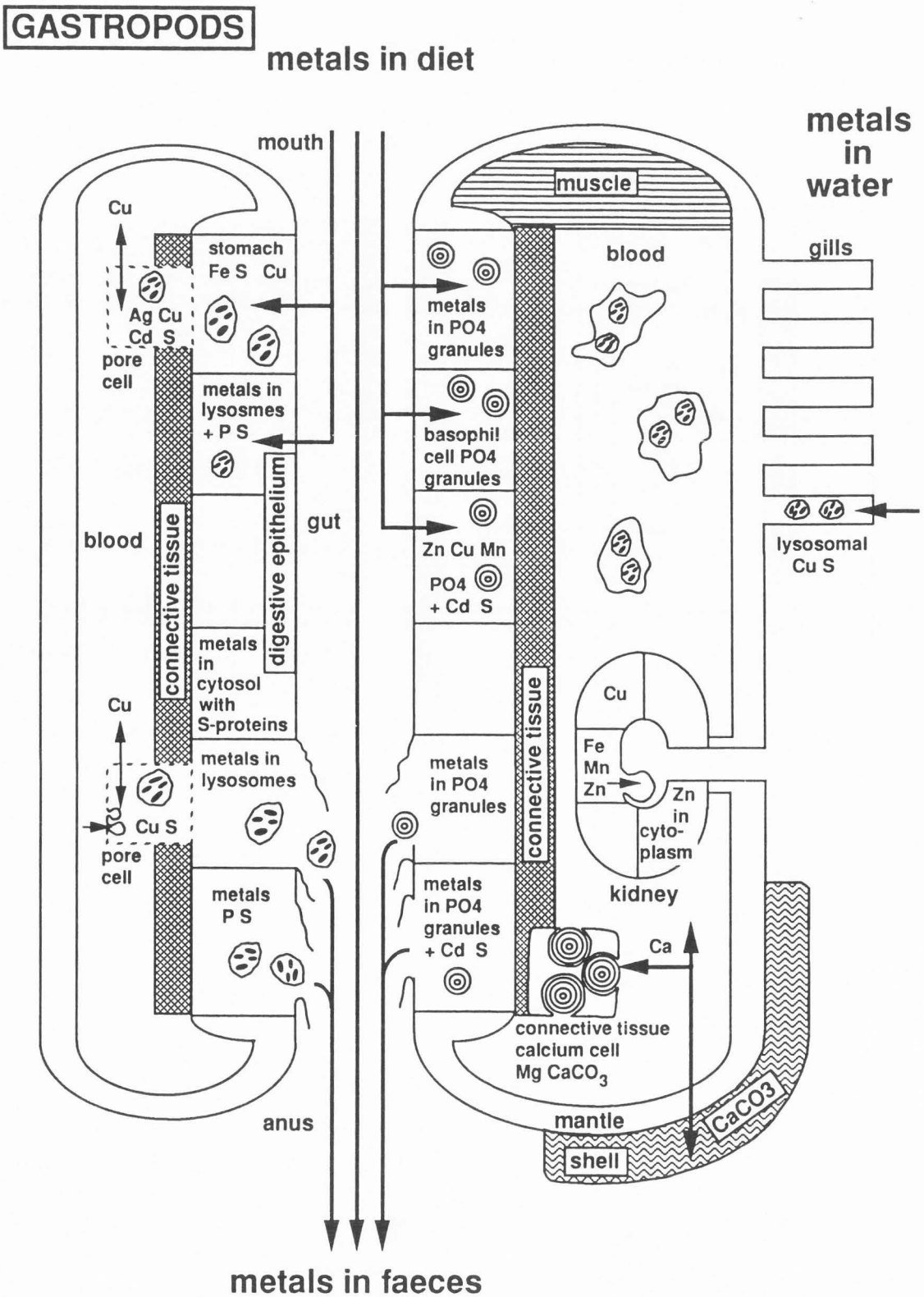

Fig. 3. Marine gastropods. Sites of metal accumulation occur in the gills and kidney, although most recordings have been made on intracellular inclusions in the gut diverticulum or hepatopancreas where metals are taken up and excreted.

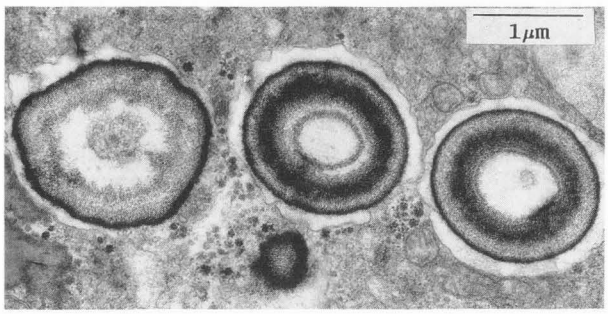

Fig. 4. Cerithium vulgatum: section of digestive gland showing mineralized phosphate granules.
Figs 4 and 5 reproduced from Nott and Nicolaidou, 1989b with permission.

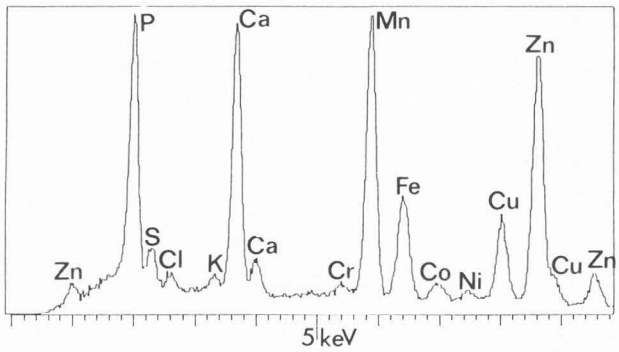

Fig. 5. Cerithium vulgatum: X-ray analysis of phosphate granules containing heavy metals. Compare with Figs. 9 and 10 which show phosphate granules without heavy metals.

Table 4 Review papers on the cytology of metals which cite X-ray microanalytical work. Other reviews appear in Tables 1-3 marked (R).

Review	Metals	Animals
Brown, 1982	Metal containing granules	Invertebrates
Bryan, 1984	Metal pollution	Marine organisms
Fowler, 1987	Intra-cellular metals	Aquatic organisms
Fowler et al., 1981	Intra-cellular metals	Estuarine organisms
George, 1980	Metal metabolism	Marine bivalves
George, 1982	Metal detoxication	Aquatic animals
George & Viarengo, 1985	Metal detoxification	Mussels
Martoja et al., 1975	Ecological metals	Various organisms
Rainbow, 1988	Metal accumulations	Decapods
Ray & McLeese, 1987	Cadmium	Marine organisms
Simkiss, 1976	Biomineralization	Invertebrates
Simkiss et al., 1982	Metal detoxification	Molluscs
Taylor & Simkiss, 1984	Biomineralization	Invertebrates
Taylor et al., 1986	Intracellular granules	Invertebrates
Viarengo, 1989	Metal metabolism	Marine invertebrates

Table 5 Additional papers which utilize XRMA for studying metals in marine organisms

Reference	Metal(s)	Animals/tissue
Bell et al., 1982	Vanadium & sulphur	Ascidian blood cells
Bone et al., 1987	Ionic analysis	Chaetognath body fluids
Gibbs & Bryan, 1980	Cu	Polychaete jaws
Gibbs & Bryan, 1984	$CaPO_4$	Polychaete skeleton
Gibbs et al., 1981	Cu	Polychaete
Gooday & Nott, 1982	$BaSO_4$,Sr (Intracellular)	Deep-sea protozoans
Gupta & Hall, 1984	Ca, Cu, Zn	Sea anemone
Nott & Parkes, 1975	Ca secretion	Polychaete
Pirie & Bell, 1984	Vanadium & sulphur	Ascidian blood
Pirie et al., 1985	Cu, Zn	Polychaete
Rowley, 1982	Vanadium	Ascidian blood cells
Southward, 1982	Zn, Cu, Ca, P	Pogonophora
for seaweed refs see: Pedersen & Roomans, 1983	Br, I	Brown seaweed

suggests that the detoxifying system which operates in the prey continues to be effective within the carnivore. This idea has been tested at Plymouth by an XRMA examination of metal from three local invertebrate predator/prey relationships (Fig. 6). The first experiment was carried out by taking the digestive gland loaded with granules from the grazing winkle *Littorina littorea* and feeding it to the carnivorous whelk *Nassarius reticulatus*. Subsequently, faecal pellets were collected from the whelk. The winkle digestive gland and the whelk faecal pellets were both squashed onto graphite specimen stubs and examined in the SEM. The glands and the pellets

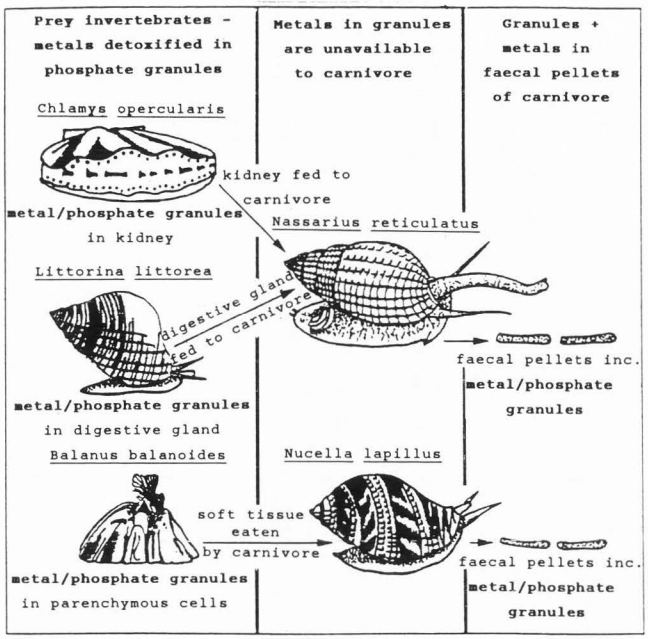

Fig. 6. Predator/prey relationships: metal containing phosphate granules are produced in tissues of the prey animals (left): tissues are fed to carnivores (centre): granules pass through gut of carnivore and appear in faecal pellets (right).

Reproduced from Nott and Nicolaidou, 1990 with permission.

produced masses of granules (Figs 7 & 8), and both produced a similar Mg/Ca/P X-ray spectrum (Figs 9 & 10) showing that the granules synthesised in the winkle were passing through the gut of the carnivore. The next stage was to dose the winkles with 1 ppm Zn in the seawater for 16 days to load metal into the granules in the digestive gland. The digestive gland was again fed to the whelk and faecal pellets were sampled in due course. the winkle gland and whelk pellets produced granules with X-ray spectra which included a peak for zinc (Figs 11 & 12); the experiment was repeated with manganese with a similar result. These observations provide circumstantial evidence that zinc and manganese were strongly bound in the granules and unavailable to the digestive system of the carnivore.

Bivalves produce phosphate granules in the kidney and these accumulate heavy metals. Kidney tissue was taken from the queen scallop *Chlamys opercularis* and fed to the whelk. Again, the granules passed through the gut of the whelk and in the faecal pellets produced XRMA spectra similar to those in the kidney (Figs 13 & 14).

Metal-binding phosphate granules also occur in barnacles (Walker et al. 1975 a & b) which are the natural prey of the dogwhelk *Nucella lapillus*. Granules extracted from barnacles and from faecal pellets of the dogwhelk gave similar spectra.

Although these results indicate that the phosphate granules can pass through the gut of a carnivore without loss of metals, the XRMA spectra of solid spherical objects like granules cannot be processed quantitatively. Differential variations in peak height due to surface geometry are high. To take the investigation further the tissues containing granules and the faecal pellets have been frozen, freeze dried, embedded and sectioned for analysis by STEM/XRMA at 200kV. Spectra are generated from the flat surfaces of the granules and X-ray counts are taken for the peaks and selected regions of background. A simple computer programme calculates the peak-minus-background integrals for the peaks. These are used to produce ratios of elemental counts which indicate changes in the constitution of the granules.

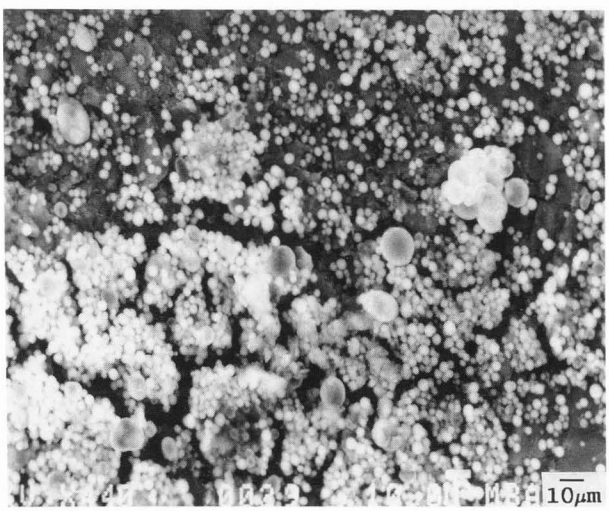

Fig. 7. Nassarius reticulatus: masses of granules in faecal pellet squashed on graphite stub

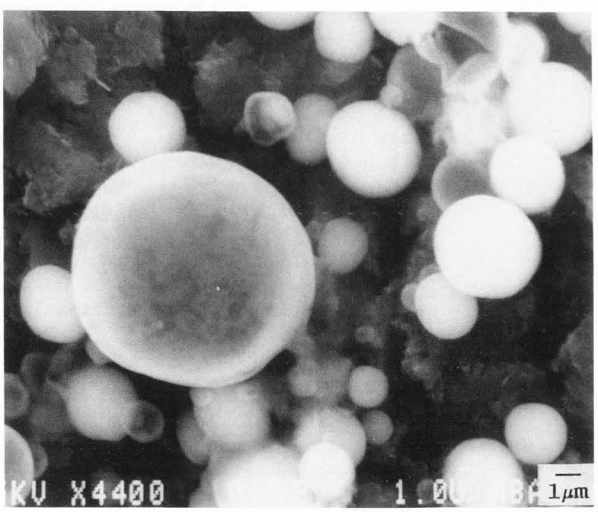

Fig. 8. Nassarius reticulatus: detail of granules in Fig. 7. The smaller dense granules are the phosphate type

Figs 7-14 reproduced from Nott and Nicolaidou, 1990 with permission

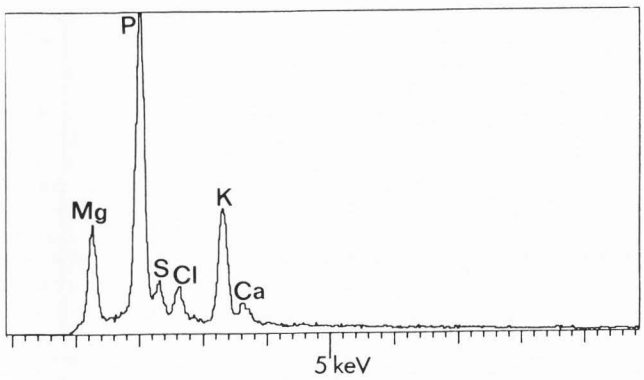

Fig. 9 *Littorina littorea* (winkle): analysis of phosphate granule in digestive gland (compare with Fig. 10)

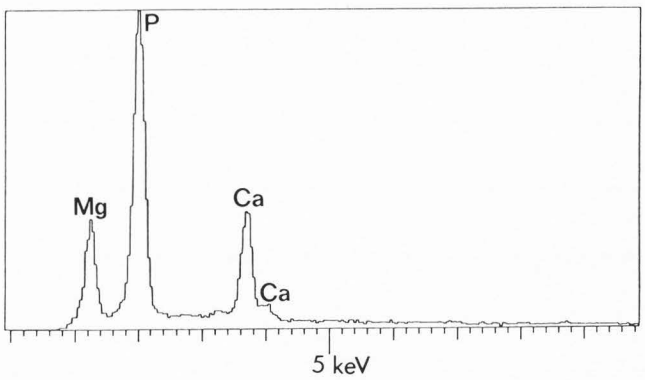

Fig. 10 *Nassarius reticulatus* (carnivorous whelk): analysis of phosphate granule in faecal pellet: granule is derived from winkle and has passed through gut of the whelk (compare with Fig. 9)

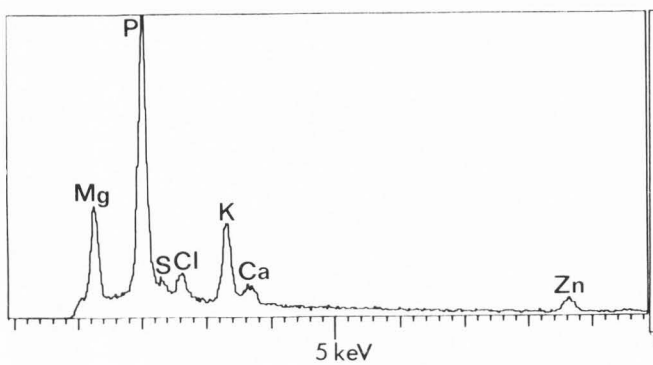

Fig. 11 *Littorina littorea*: analysis of phosphate granule containing zinc (compare with Fig. 12)

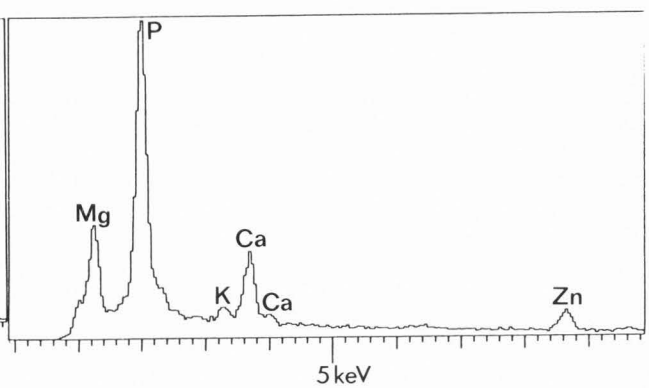

Fig. 12 *Nassarius reticulatus*: analysis of phosphate granule in faecal pellet: the zinc incorporated in the pellet by the winkle has not been removed by the digestive system of the whelk (cf with Fig. 11)

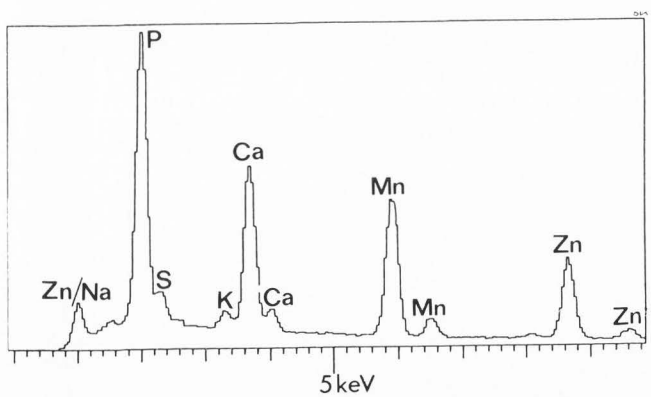

Fig. 13 *Chlamys operularis* (scallop): analysis of phosphate granule in kidney (compare with Fig. 14)

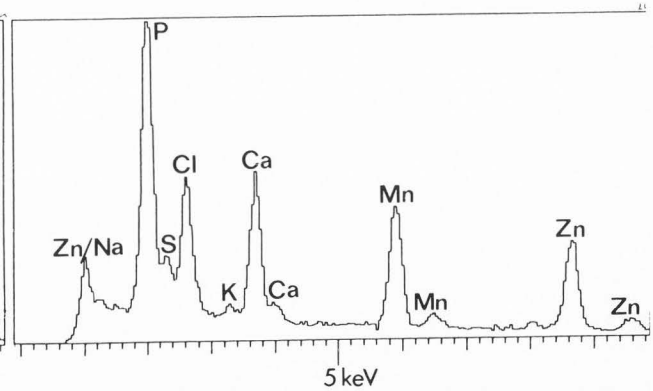

Fig. 14 *Nassarius reticulatus*: analysis of phosphate granule in faecal pellet: granule is derived from scallop kidney and has passed through the gut of the whelk (compare with Fig. 13)

Cytology of metal interactions

In another investigation the combination of cryopreparation and the analytical resolution and sensitivity of the STEM/XRMA system have demonstrated a precise intracellular location of cadmium (Nott and Langston, 1989). In μm thick sections of winkle digestive gland, the phosphate granules were analysed in a transect across the diameter (Fig. 15). Cadmium, in association with sulphur, is confined to the membrane which encloses the granule. It is conceivable that this cadmium binding could affect the efficiency of transport of metals across the membrane before they can be incorporated in the granule and bound to phosphate radicals.

It is known from metal uptake work that there are both synergistic and antagonistic interactions between cadmium and other elements. Clearly, the relationship between membrane-associated cadmium and the composition of the granules can be investigated further by XRMA and provide some basic mechanistic interpretation of the cytology and inorganic biochemistry of heavy metal reactions and interactions.

Summary

The application of XRMA to pollution research is providing information on the effects of metals on marine organisms and is demonstrating that detoxification of metals by a particular species can reduce the availability and toxicity of metals to other organisms. XRMA can be tuned to observe the complex interactions which operate at all levels within and between the biota and polluted environments.

Acknowledgements

The author wishes to acknowledge the work of all collaborators during the last twenty years and to mention in particular Dr. Artemis Nicolaidou (University of Athens, Greece), Linda Mavin and Keith Ryan (Plymouth Marine Laboratory, UK).

References

Al-Mohanna SY, Nott JA (1986a) The accumulation of metals in the hepatopancreas of the shrimp *Penaeus semisulcatus* De Haan (Crustacea:Decapoda) during the moult cycle. Proc. First Arabian Gulf Conf. on Environment and Pollution. (Eds) Halwagy R, Clayton D and Behbehani M. Kuwait University, Faculty of Science, PO Box 5969, Kuwait, pp 195-209.

Al-Mohanna SY, Nott JA (1986b) B-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea:Decapoda). J mar biol Ass UK 66 403-414.

Al-Mohanna SY, Nott JA (1987) R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea:Decapoda). Mar Biol 95 129-137.

Al-Mohanna SY, Nott JA (1989) Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea:Decapoda) during the moult cycle. Mar Biol 101 535-544.

Ballan-Dufrancais C, Jeantet A-Y, Halpern S (1982) Localisation intracellulaire par microanalyse X de métaux et de métalloides dans la glande digestive d'un Mollusque Bivalve *Pecten maximus*. Implications des processus de digestion. C R Acad Sc Paris 294 673-678.

Ballan-Dufrancais C, Jeantet A-Y, Feghali C, Halpern S (1985) Physiological features of heavy metal storage in bivalve digestive cells and amoebocytes: EPMA and factor analysis of correspondences. Biol Cell 53 283-292.

Bell MV, Pirie BJS, McPhail DB, Goodman BA, Falk-Petersen I-B, Sargent JR (1982) Contents of vanadium and sulphur in the blood cells of *Ascidia mentula* and *Ascidiella aspersa*. J mar biol Ass UK 62 (3) 709-716.

Bone Q, Brownlee C, Bryan GW, Burt GR, Dando PR, Liddicoat MI, Pulsford AL, Ryan KP (1987) On the differences between the two 'indicator' species of chaetognath *Sagitta setosa* and *S. elegans*. J mar biol Ass UK 67 (3) 545-560.

Bouquegneau JM, Martoja M (1982) La teneur en cuivre et son degré de complexation chez quatre Gastéropodes marins. Données sur le cadmium et le zinc. Oceanol Acta 5 (2) 219-228.

Bouquegneau JM, Martoja M, Truchet M (1984) Heavy metal storage in marine animals under various environmental conditions. In: Toxins, Drugs and Pollutants in Marine Animals. (Eds) Bolis L, Zadunaisky J, Gilles R. Springer Verlag, Berlin, Heidelberg, pp 147-160.

Brown BE (1982) The form and function of metal-containing 'granules' in invertebrate tissues. Biol Rev 57 621-667.

Bryan GW (1984) Pollution due to heavy metals. Mar Ecol 5 (3) 1289-1431.

Bryan GW, Hummerstone LG, Ward E (1986) Zinc regulation in the lobster *Homarus gammarus*: importance of different pathways of absorption and excretion. J mar biol Ass UK 66 175-199.

Carmichael NG, Fowler BA (1981) Cadmium accumulation and toxicity in the kidney of the Bay Scallop *Argopecten irradians*. Mar Biol 65 35-43.

Carmichael NG, Squibb KS, Fowler BA (1979) Metals in the molluscan kidney: a comparison of two closely related bivalve species (Argopecten) using X-ray microanalysis and atomic absorption spectroscopy. J Fish Res Bd Can 36 1149-1155.

Chassard-Bouchaud C (1983) Rôle des lysosomes et des sphérocristaux dans le phénomène de concentration de l'uranium chez la Moule *Mytilus edulis* (L.) Microanalyse par spectrographie des rayons X. C R Acad Sc Paris 296 581-586.

Chassard-Bouchaud C (1985) Bioaccumulation de métaux stables et radioactifs par les organismes benthiques de la baie de Seine: aspects structuraux, ultrastructuraux et micro-analytiques. Cah de Biol Mar 26 63-85.

Chassard-Bouchaud C, Hallegot P (1984) Bioaccumulation de lanthane par des Moules *Mytilus edulis* (L) récoltées sur les côtes francaises. Microanalyse par spectrographie des rayons X et par émission ionique secondaire. C R Acad Sc Paris 298 (20) 567-572.

Chassard-Bouchaud C, Fiala-Medioni A, Galle P (1986) Etude microanlytique de *Bathymodiolus* sp. (Mollusque Lamellibranche Mytilidae) provenant des sources hydrothermales de la Ride du Pacifique oriental. Donées préliminaires. C R Acad Sc Paris 302 (4) 117-124.

Chassard-Bouchaud C, Galle P, Escaig F (1985) Mise en évidence d'une contamination par l'argent

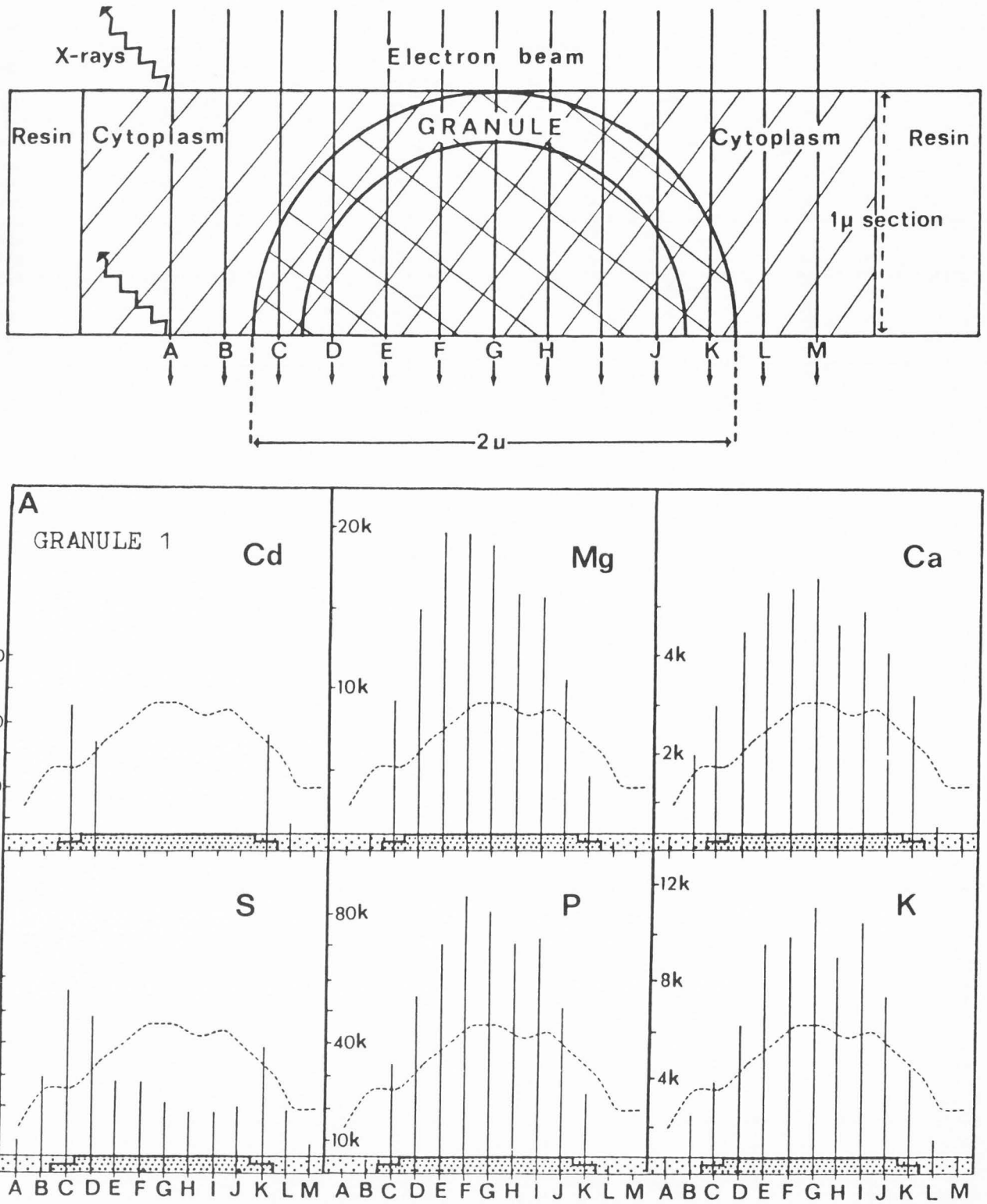

Figure 15. Littorina littorea (top) Diagrammatic section through a phosphate granule with a transect of XRMA points A-M at 0.2 μm intervals; (bottom) 100 sec counts (p-b) for 13 points on the transect for 6 elements. Cadmium is confined to the high S margins of the granule. Reproduced form Nott and Langston, 1989, with permission.

et le plomb de l'Huitre *Crassostrea gigas* et de la Moule *Mytilus edulis* dans les eaux côtiéres françaises. Etude microanalytique par émission ionique secondaire. C R Acad Sc Paris 300 (1) 3-8.

Chassard-Bouchaud C, Boutin JF, Hallegot P, Galle P (1989) Chromium uptake, distribution and loss in the mussel *Mytilus edulis*: a structural, ultrastructural and microanalytical study. Dis aquat Org 7 117-136.

Coombs TL, George SG (1978) Mechanisms of immobilization and detoxication of metals in marine organisms. In: Physiology and behaviour of marine organisms. (Eds) McLusky DS and Berry AJ. Oxford: Pergamon Press, pp 179-187.

Doyle LJ, Blake NJ, Wood CC, Yevich P (1978) Recent biogenic phosphorite: concretions in mollusk kidneys. Science 199 1431-1433.

Fowler BA (1987) Intracellular compartmentation of metals in aquatic organisms; roles in mechanisms of cell injury. Environ Hlth Perspect 71 121-128.

Fowler BA, Carmichael NG, Squibb KS, Engel DW (1981) Factors affecting trace metal uptake and toxicity to estuarine organisms. II. Cellular mechanisms. In: Biological Monitoring of Marine Pollutants. (Eds) Vernberg FJ, Calabrese A, Thurberg FP, Vernberg WB. Academic Press, New York, pp 145-163.

Fowler BA, Wolfe DA, Hettler WF (1975) Mercury and iron uptake by cytosomes in mantle epithelial cells of quahog clams (*Mercenaria mercenaria*) exposed to mercury. J Fish Res Bd Can 32 1767-1775.

George SG (1980) Correlation of metal accumulation in mussels with the mechanisms of uptake, metabolism and detoxification: a review. Thal Jugo 16 (2-4) 347-365.

George SG (1982) Subcellular accumulation and detoxication of metals in aquatic animals. In: Physiological mechanisms of marine pollutant toxicity. (Eds) Vernberg WB, Calabrese A, Thurberg FP, Vernberg FJ. Academic Press, pp 3-52.

George SG, Pirie BJS (1979) The occurrence of cadmium in sub-cellular particles in the kidney of the marine mussel, *Mytilus edulis*, exposed to cadmium. The use of electron microprobe analysis. Biochem Biophys Acta 580 234-244.

George SG, Pirie BJS (1980) Metabolism of zinc in the mussel *Mytilus edulis* (L.): a combined ultrastructural and biochemical study. J mar biol Ass UK 60 575-590.

George SG, Viarengo A (1985) A model for heavy metal homeostasis and detoxication in mussels. In: Marine pollution and physiology: recent advances. (Eds) Vernberg FJ, Thurberg FP, Calabrese A, Vernberg WB. The Belle W. Baruch Library in Marine Science No 13. University of Southern California Press, pp 125-139.

George SG, Pirie BJS, Coombs TL (1976) The kinetics of accumulation and excretion of ferric hydroxide in *Mytilus edulis* (L.) and its distribution in the tissues. J exp mar Biol Ecol 23 71-84.

George SG, Pirie BJS, Coombs TL (1977) Absorption, accumulation and excretion of iron-protein complexes by *Mytilus edulis* (L.) In: Int Conf on heavy metals in the environment. Proc Vol 2. (Ed.) Hutchinson TC. University of Toronto, pp 887-900.

George SG, Pirie BJS, Cheyne AR, Coombs TL, Grant PT (1978) Detoxication of metals by marine bivalves: An ultrastructural study of the compartmentation of copper and zinc in the oyster *Ostrea edulis*. Mar Biol 45 147-156.

George SG, Pirie BJS, Coombs TL (1980) Isolation and elemental analysis of metal-rich granules from the kidney of the scallop *Pecten maximus* (L.). J exp mar Biol Ecol 42 143-156.

Gibbs PE, Bryan GW (1980) Copper - the major component of glycerid polychaete jaws. J mar biol Ass UK 60 205-214.

Gibbs PE, Bryan GW (1984) Calcium phosphate granules in muscle cells of *Nephthys* (Annelida, Polychaeta) - a novel skeleton? Nature 310 (5977) 494-495.

Gibbs PE, Bryan GW, Ryan KP (1981) Copper accumulation by the polychaete *Melinna palmata*: an antipredation mechanism? J mar biol Ass UK 61 (3) 707-722.

Gooday AJ, Nott JA (1982) Intracellular barite crystals in two Xenophyophores, *Aschemonella ramuliformis* and *Galatheammina* sp. (Protozoa:Rhizopoda) with comments on the taxomony of *A. ramuliformis*. J mar biol Ass UK 62 595-605.

Greaves GN, Simkiss K, Taylor M, Binsted N (1984) The local environment of metal sites in intracellular granules investigated by using X-ray absorption spectroscopy. Biochem J 221 855-868.

Gupta BL, Hall TA (1984) Role of high concentrations of Ca, Cu and Zn in the maturation and discharge in situ of sea anemone nematocysts as shown by X-ray microanalysis of cryosections. In : Toxins, Drugs and Pollutants in Marine Animals. (Eds) Bolis L, Zadunaisky J, Gilles R. Springer Verlag, pp 77-95.

Hopkin SP, Nott, JA (1979) Some observations on concentrically structured, intracellular granules in the hepatopancreas of the shore crab *Carcinus maenas* (L.) . J mar biol Ass UK 59 867-877.

Hopkin SP, Nott JA (1980) Studies on the digestive cycle of the shore crab *Carcinus maenas* (L.) with special reference to the B-cells in the hepatopancreas. J mar biol Ass UK 60 891-907.

Icely JD, Nott JA (1980) Accumulation of copper within the 'hepatopancreatic' caeca of *Corophium volutator* (Crustacea:Amphipoda). Mar Biol 57 193-199.

Icely JD, Nott JA (1985) Feeding and digestion in *Corophium volutator* (Crustacea:Amphipoda). Mar Biol 89 183-195.

Koulish S (1976) Organization of 'special' parenchymal cells underlying the midgut in some barnacles. J exp mar Biol Ecol 23 155-170.

Marshall AT, Talbot V (1979) Accumulation of cadmium and lead in the gills of *Mytilus edulis*: X-ray microanalysis and chemical analysis. Chem Biol Interact 27 111-123.

Martoja M, Tue VT, Elkaim B (1980) Bio-accumulation du cuivre chez *Littorina littorea* (L.) (Gastéropode Prosobranche): signification physiologique et écologique. J exp mar Biol Ecol 43 251-270.

Martoja M, Bouquegneau JM, Truchet M, Martoja R (1985) Recherche de l'argent chez quelques mollusques marins, dulcicoles et terrestres. Formes chemiques et localisation histologique. Vie Milieu 35 (1) 1-13.

Martoja R. Martin J-L (1985) Recherche des mécanismes de détoxication du cadmium par l'Huitre *Crassostrea gigas* (Mollusque,Bivalve). I. Mise en evidence d'une protéine sulfhydrilée de complexation du métal dans les amoebocytes à zinc et cuivre. C R Acad Sc Paris 300 (15) 549-554.

Martoja R, Alibert J, Ballan-Dufrancais C, Jeantet A-Y, Lhonore D, Truchet M (1975) Microanalyse et ecologie. J Microsc Biol Cell 22 441-448.

Mason AZ, Nott JA (1980) A rapid, routine technique for the X-ray microanalysis of micro-incinerated cryosections: An SEM study of inorganic deposits in tissues of the marine gastropod *Littorina littorea* (L.). J Histochem Cytochem 28 (12) 1301-1311.

Mason AZ, Nott JA (1981) The role of intracellular biomineralized granules in the regulation and detoxification of metals in gastropods with special reference to the marine prosobranch *Littorina littorea*. Aquat Toxicol 1 239-256.

Mason AZ, Simkiss K (1982) Sites of mineral deposition in metal-accumulating cells. Exp Cell Res 139 383-391.

Mason AZ, Simkiss K, Ryan KP (1984) The ultrastructural localization of metals in specimens of *Littorina littorea* collected from clean and polluted sites. (Menai Bridge [clean] and Restronguet Creek on Fal Estuary [polluted]). J mar biol Ass UK 64 699-720.

Mauri M, Orlando E (1982) Experimental study on renal concretions in the wedge shell *Donax trunculus* L. J exp mar Biol Ecol 63 47-57.

Nott JA, Langston WJ (1989) Cadmium and the phosphate granules in *Littorina littorea*. J mar biol Ass UK 69 219-227.

Nott JA, Mavin LJ (1986) Adaptation of a quantitative programme for the X-ray analysis of solubilized tissue as microdroplets in the transmission electron microscope: application to the moult cycle of the shrimp *Crangon crangon* (L.). Histochem J 18 507-518.

Nott JA, Nicolaidou A (1989a) Metals in gastropods - metabolism and bioreduction. Mar Envir Res 28 201-205.

Nott JA, Nicolaidou A (1989b) The cytology of heavy metal accumulations in the digestive glands of three marine gastropods. Proc R Soc Lond 237B 347-362.

Nott JA, Nicolaidou A (1990) Transfer of metal detoxification along marine food chains. J mar biol Ass UK 70 905-912.

Nott JA, Parkes KR (1975) Calcium accumulation and secretion in the serpulid polychaete *Spirorbis spirorbis* L. at settlement. J mar biol Ass UK 55 911-923.

Nott JA, Corner EDS, Mavin LJ, O'Hara SCM (1985) Cyclical contributions of the digestive epithelium to faecal pellet formation by the copepod *Calanus helgolandicus*. Mar Biol 89 271-279.

Pedersén M, Roomans GM (1983) Ultrastructural localization of bromine and iodine in the stipes of *Laminaria digitata* (Huds.) Lamour., *Laminaria saccharina* (L.) Lamour and *Laminaria hyperborea* (Gunn.) Fosl. Bot Mar 26 113-118.

Pirie BJS, Bell MV (1984) The localization of inorganic elements, particularly vanadium and sulphur, in haemolymph from the ascidians *Ascidia mentula* (Müller) and *Ascidiella aspersa* (Müller). J exp mar Biol Ecol 74 187-194.

Pirie BJS, Fayi L, George S (1985) Ultrastructural localisation of copper and zinc in the polychaete *Nereis diversicolor*, from a highly contaminated estuary. Mar environ Res 17 197-198.

Pirie BJS, George SG, Lytton DG, Thomson JD (1984) Metal-containing blood cells of oysters: Ultrastructure, histochemistry and X-ray microanalysis. J mar biol Ass UK 64 115-123.

Rainbow PS (1987) Heavy metals in barnacles. In: Barnacle Biology. [Crustacean Issues 5] (Ed.) Southward AJ. AA Balkema, Rotterdam, pp 405-417.

Rainbow PS (1988) The significance of trace metal concentrations in decapods. Symp zool Soc Lond 59 291-313.

Ray S, McLeese DW (1987) Biological cycling of cadmium in marine environment. In: Cadmium in the aquatic environment. (Eds) Nriagu JO, Sprague JB. John Wiley & Sons, pp 199-229.

Rowley AF (1982) The blood cells of *Ciona intestinalis*: an electron probe X-ray microanalytical study. J mar biol Ass UK 62 (3) 607-620.

Ryan KP, Purse DH (1984) Rapid freezing: specimen supports and cold gas layers. J Microsc 136 RP5-RP6.

Ryan KP, Purse DH, Robinson SG, Wood JW (1987) The relative efficiency of cryogens used for plunge-cooling biological specimens. J Microsc 145 89-96.

Ryan KP, Bald WB, Neumann K, Simonsberger P, Purse DH, Nicholson DN (1990) Cooling rate and ice-crystal measurement in biological specimens plunged into liquid ethane, propane and Freon 22. J Microsc 158 365-378.

Schulz-Baldes M (1977) Lead transport in the common mussel *Mytilus edulis* . In: Proc 12th European Marine Biology Symposium. (Eds) McLusky DS, Berry AJ. Pergamon Press, pp 211-218.

Simkiss K (1976) Intracellular and extracellular routes in biomineralization. Symp Soc exp Biol 30 423-444.

Simkiss K (1979) Metal ions in cells. Endeavour (new series) 3 (1) 2-6.

Simkiss K (1984) The karyotic mineralization window (KMW). Amer Zool 24 847-856.

Simkiss K, Mason AZ (1983) Metal Ions: Metabolic and toxic effects. In: The Mollusca, Vol 2. (Ed.) Hochachka PW. Academic Press, pp 101-164.

Simkiss K, Mason AZ (1984) Cellular responses of molluscan tissues to environmental metals. Mar envir Res 14 103-118.

Simkiss K, Taylor M, Mason AZ (1982) Metal detoxication and bioaccumulation in molluscs. Mar Biol Lett 3 187-201.

Southward EC (1982) Bacterial symbionts in Pogonophora. J mar biol Ass UK 62 (4) 889-906.

Taylor MG, Simkiss K (1984) Inorganic deposits in invertebrate tissues. Envir Chem 3 102-138.

Taylor M, Simkiss K, Greaves GN (1986) Amorphous structure of intracellular mineral granules. Biochem Soc Trans 14 (3) 549-552.

Taylor M, Simkiss K, Greaves GN, Harries J (1988) Corrosion of intracellular granules and cell death. Proc R. Soc Lond 234B, 463-476.

Thomas PG, Ritz DA (1986) Growth of zinc granules in the barnacle *Elminius modestus*. Mar

Biol 90 255-260.

Thomson JD, Pirie BJS, George SG (1985) Cellular metal distribution in the Pacific oyster *Crassostrea gigas* (Thun.) determined by quantitative X-ray microprobe analysis. J exp mar Biol Ecol 85 37-45.

Viarengo A (1989) Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the cellular level. Rev aquat Sci 1 (2) 295-317.

Walker G (1977) "Copper" granules in the barnacle *Balanus balanoides*. Mar Biol 39 343-349.

Walker G, Rainbow PS, Foster P, Crisp DJ (1975a) Barnacles: Possible indicators of zinc pollution? Mar Biol 30 57-65.

Walker G, Rainbow PS, Foster P, Holland DL (1975b) Zinc phosphate granules in tissue surrounding the midgut of the barnacle *Balanus balanoides*. Mar Biol 33 161-166.

Discussion with Reviewers

L Sacko-Goad: Is there any evidence that there is an increased concentration of metals in the gut of predators that ingest heavy metal laden prey?
Author: In a polluted region of the Mediterranean the carnivore *Murex trunculus* accumulates metals from the gastropod *Cerithium vulgatum* (Nott and Nicolaidou 1989b). However the carnivore does not accumulate the full range of metals from the prey and the concentrations in the digestive gland are lower in the carnivore than the prey. The exception is copper which occurs at higher concentration in the predator.

The final metal accumulations in the predator must depend upon the total metals present in the prey and the bioavailabilities in the various tissues. In addition, the levels in the carnivore will be the net balance of continuous uptake and excretion.

AJ Morgan: You appear pessimistic about the ability of XRMA to provide fully quantitative and reliable, biological data. Would you define what you consider to be the major constraints?
Author: In the SEM the constraints on quantitative XRMA are the effects of surface geometry on beam penetration and X-ray emission and detection. In the TEM and STEM the constraints are again geometrical but generally they can be controlled. However, there remain unexplained discrepancies like the variations in analyses across the surfaces of sectioned 2 μm diameter granules which are related consistently with respect to the direction of the detector. Most biological XRMA projects are subject to the constraints imposed upon specimen preparation by the solubility of elements.

AJ Morgan: You show that Cd and S are associated with the limiting membrane of the phosphate granules in the winkle digestive gland. Is Cd bound to a S-donating ligand in the membrane or do the granules possess a peripheral Cd-S component? Maybe the Cd and S peaks are generated from Cd-metallothioneins in the cytoplasm by electrons scattered from the mineralized granules?
Author: We ask the same questions and hope to have an answer after more experiments.

AZ Mason: The proposal for the progressive bioreduction of metals up the food chain is an intriguing and novel concept and the presented evidence for this process appears to be extremely strong. Nevertheless, would the author agree that it is important to distinguish whether the analysed granules are derived from unassimilated food or from the basophil cells of the carnivorous molluscs?
Author: The granules in the faecal pellets are not derived from the basophil cells of the carnivore. The granules in the basophil cells produce spectra which are distinctive in each carnivorous species and they do not match the spectra derived from the granules of the prey.

AZ Mason: As the author correctly points out, Cd in most cells is either associated with metallothionein-like proteins or alternatively with lysosomal bodies which are presumed to be derived from the degradation of these proteins. The presence of Cd associated with suphur at the membrane delineating the granules therefore raises a number of interesting questions: do high power electron micrographs show the presence of lysosomal inclusions in this area? If not, are the metals sequestered to metallothionein, and if so how is this cytoplasmic protein localized without any apparent compartmentation? Could the presence of the Cd and S on the surface of the granules be a drying artifact brought about by en bloc drying and resin impregnation and by the subsequent collapse, concentration and accretion of dry/drying cytoplasm onto the surface of the granules? Have similar results been obtained by viewing hydrated cryosections?
Author: There are no lysosomal inclusions in the areas analysed. In animals not dosed with cadmium the margins of the granules produce a similar peak for sulphur which is not associated with cadmium. It can be suggested, therefore, that when cadmium is present, it is associated with a non-induced, indigenous, high-sulphur protein or that the cadmium and sulphur are not associated chemically.

Hydrated sections have not been analysed. However, freeze-dried sections have been viewed on a cold stage at -175°C and the spectra are similar to those produced with a conventional stage. This indicates that the cadmium and sulphur are not lost differentially into the high vacuum of the EM under the action of the electron beam.

K Simkiss: XRMA focuses investigations on discrete accumulations such as granules and lysosomes that often refer to the end products rather than the processes of metal metabolism.
Author: This point is accepted and it arises mainly from the limit of detection for an element to about 0.1% of the mass of the volume analysed. Thus, in the blood of marine invertebrates metals can be detected in accumulations carried by the lymphocytes but they cannot be detected when dispersed in the plasma. In general, however, the chemical characteristics of accumulations together with their temporal and spatial distribution can contribute substantially to investigations into metal metabolism.

K Simkiss: The statement that the reactions of metals "are chemically simple but biologically complex" refers to the concept of the group a (hard) and group b (soft) metals which has helped biologists understand the basis of ligand binding. Now, it is opportune to look further into the co-ordination and stereo-chemistry of biological systems. For example, the localization of cadmium on the membrane of the phosphate granule in association with a sulfur signal is to be expected for a group b metal. However, in a number of epithelia it has been suggested that cadmium may be transported with calcium because of similarities in atomic size. XRMA would not pick this up.

Author: Apart from the co-ordination and stereo-chemistry of cadmium, only limited examples of intracellular accumulations have been detected by XRMA. Cadmium is easily removed from tissue by chemical fixation which makes cryopreparation essential. Also, much of it is bound to dispersed cytosolic metalloproteins and this reduces the mass fraction of the element below the detection level of XRMA. These properties are associated with its high toxicity and extended retention within tissues. Other pollutant metals are detoxified as insoluble intracellular accumulations which are excreted during cell turnover. These accumulations are readily detectable by XRMA.